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TRANSCRIPTIONAL AND EPIGENETIC REGULATION OF GENE EXPRESSION BY ARSENIC IN CANCER AND NORMAL CELLS

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Transcriptional and epigenetic regulation of gene expression by arsenic in cancer and normal cells

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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"…καὶ ἐπειδὴ πρὸς τὸ φῶς ἔλθοι, αὐγῆς ἂν ἔχοντα τὰ ὄμματα μεστὰ ὁρᾶν οὐδ' ἂν ἓν δύνασθαι τῶν νῦν λεγομένων ἀληθῶν;"

Πλάτων, Πολιτεία

"…and when he came out into the light, that his eyes would be filled with its beams so that he would not be able to see even one of the things that we call real?"

Plato, Republic

To my beloved husband

ABSTRACT

Inorganic arsenic is a toxic metalloid that occurs naturally on the earth's crust. Millions of people worldwide are exposed to inorganic arsenic via drinking water, and more recently also via contaminated food, in particular rice. Many studies have focused on exploring the adverse health effects of arsenic as well as its mode of action once entering the body. Still, little is known about the intracellular processes that drive arsenic toxicity. In the present thesis we investigated the effects of arsenic on transcriptional and epigenetic processes both in normal and cancer cell lines.

In order to understand how exogenous compounds affect transcriptional regulators such as MAML1, which is involved in many different signaling pathways and has been related to developmental processes and human diseases (e.g. cancer), it's important to understand which cellular processes regulate the protein levels. Consequently, we studied the transcriptional co-activator MAML1 and the ubiquitination and degradation process. We show that MAML1 protein levels are regulated via ubiquitination and that this process is enhanced by p300 and repressed by N1ICD. On top of that, we also investigated MAML1 involvement in cell proliferation and epigenetic regulation as well as arsenic effect on MAML1 expression and kidney cell proliferation. We show that MAML1 interacts with DNMT1 and PCNA, both members of the DNMT1-PCNA-HDAC2 repressive complex, which is involved in epigenetic regulation. We further report that arsenic decreases kidney cell proliferation and we suggest this occurs via MAML1 downregulation.

In order to further explore the effects of arsenic on the epigenome, we studied arsenic exposure in relation to the post-translational histone modifications (PTHMs) H3K9me3 and H3K9Ac in lymphocytes isolated from exposed individuals as well as *in vitro* in cell culture system. We report arsenic-related changes in H3K9me3 epigenetic mark in CD4+ lymphocytes isolated from the arsenic exposed individuals and changes in H3K9Ac in *in vitro* cultured T lymphoblasts exposed to arsenic.

In conclusion, our data suggest that MAML1 protein levels are regulated via ubiquitination, a process that could also be targeted by arsenic and in this way influence gene expression. Moreover, we suggest that arsenic regulates MAML1 protein levels and could thereby also influence the cell signaling pathways depending on MAML1 transcriptional activity. Arsenic also targets epigenetic processes by altering the global levels of H3K9me3 and H3K9Ac in lymphocytes, which could lead to adverse health effects in the human population.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to throughout the text by their Roman numerals I-III:

I. M. Farshbaf, M. J. Lindberg, A. Truong, Z. Bevens, E. Chambers, **A. Pournara**, A. E. Wallberg and J. B. White (2015). Mastermind-Like 1 Is Ubiquitinated: Functional Consequences for Notch Signaling. *PLoS One, 10, e0134013*.

II. **A. Pournara**, T. Holmlund, Y. Lu, R. Ceder, M. Putnik, R. Grafstrom, M. Vahter and A. E. Wallberg (2014). Arsenic-induced suppression of kidney cell proliferation and the transcriptional coregulator MAML1. *Metallomics, 6, 498-504*.

III. **A. Pournara**, M. Kippler, T. Holmlund, R.Ceder, R. Grafström, M. Vahter, K. Broberg and A. E. Wallberg (2016). Arsenic alters global histone modifications in lymphocytes in vitro and in vivo. *Manuscript.*

LIST OF RELATED SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. M. Putnik, T. K. Wojdacz, A. Pournara, M. Vahter and A. E. Wallberg (2015). MS-HRM assay identifies high levels of epigenetic heterogeneity in human immortalized cell lines. *Gene, 560, 165-172*.

CONTENTS

LIST OF ABBREVIATIONS

Acetyl-CoA	Acetyl coenzyme A
Acf-1	ATP-utilizing chromatin assembly and remodeling factor 1
ADP	Adenosine diphosphate
ANPL	Acute Nonpromyelocytic Leukemia
AP-1	Activating Protein-1
APL	Acute promyelocytic leukemia
AR	Activating Protein-1
As	Arsenic
As(III)	Trivalent Arsenite
As(V)	Pentavalent Arsenate
AS3MT	Arsenic(+3)-Methyltransferase
ATLL	Adult T-cell leukemia/lymphoma
ATO	Arsenic Trioxide
ATRA	All trans retinoic acid
ATSDR	Agency for Toxic Substances and Disease Registry
BFD	Blackfoot Disease
ccRCC	Clear-Cell Renal Cell Carcinoma
CDK8	Cyclin-Dependent Kinase 8
cDNA	Complementary DNA
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
COSMIC	Catalogue of somatic mutations in cancer

CpG	Cytosine–phosphate-guanine
DMA	Dimethylated Arsenic
DNA	Deoxyribonucleic acid
DNMT1	DNA (cytosine-5-)-methyltransferase 1
DSL	Delta– Serrate–LAG2
EPA	U.S. Environmental Protection Agency
ESR1	Estrogen Receptor 1
EU	European Union
FACScan	Fluorescence-activated cell scan
FITC	Fluorescein isothiocyanate
Fz	Frizzled
Gcn5	General Control Non-Derepressible 5
GNATs	General N-Acetyltransferases
GST	Glutathione S-transferase
HA-Ub	Human influenza hemagglutinin (HA)-Ubiquitin
HAT	Histone acetyltransferase
HATs	Histone Acetyltransferases
HCC	Hepatocellular Carcinoma
HDAC2	Histone deacetylase 2
HDACs	Histone Deacetylases
HEK	Human Embryonic Kidney
HES1	Hes family bHLH transcription factor 1
HG ICP-MS	Hydride generation inductively coupled plasma mass spectrometry
HO-1	Heme Oxygenase-1

HPLC	High-performance liquid chromatography
IARC	International agency for research on cancer
ICN	Intracellular Domain
ICP-MS	Inductively coupled plasma mass spectrometry
IST	*In silico* transcriptomics
ISWI	Chromatin-remodeling complex ATPase chain Iswi
LMP1	Latent Membrane Protein-1
MDS	Myelodysplastic Syndrome
miRNA	Micro RNA
MMA	Monomethylated Arsenic
mRNA	Messenger RNA
MTS	3-[4,5,dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4- sulfophenyl]-2H-tetrazolium, inner salt
myc	V-myc avian myelocytomatosis viral oncogene homolog
N6AMT1	N(6)-adenine-specific DNA methyltransferase
NAP1	Nucleosome assembly protein 1 (NAP-1)
NECD	Notch Extracellular Domain
NF-kB	Nuclear Factor Kappa B
NFE2L2	Nuclear Factor (Erythroid-Derived 2)-Like 2
NICD	Notch Intracellular Domain
NTPs	Nucleoside triphosphates
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PI	Propidium iodide
PPAR-γ	Peroxisome Proliferator-Activated Receptor-Gamma

PTHMs	Post-Translational Histone Modifications
RAR	Retinoic Acid Receptor
Rb	Retinoblastoma
RNA	Ribonucleic acid
RPLP0	Ribosomal phosphoprotein P0
SAM	S-Adenosylmethionine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Se	Selenium
SH	Sulfhydryl
siRNA	Small interfering RNA
TP53	Tumor protein p53
TR	Thyroid Hormone Receptor
WHO	World Health Organisation
Wt-1	Wilms Tumor Protein 1
XRCC5 (Ku80)	X-ray repair complementing defective repair in chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen 80kD)

1 INTRODUCTION

This thesis includes studies on the effects of arsenic on transcriptional and epigenetic regulation in normal and cancer cells. Arsenic has long been known for its adverse effects on human health and thus studying the underlying mechanisms will provide valuable information in arsenic toxicity (Ratnaike 2003).

1.1 ARSENIC IN THE ENVIRONMENT AND HUMAN EXPOSURE

Arsenic is a metalloid found naturally on the earth's crust, where its content may vary between 2 and 3 mg/kg (Tanaka 1988; Cullen and Reimer 1989). Arsenic can be found in the environment in various oxidative states (3, 0, +3, +5), but the most common forms in natural waters are oxyanions of trivalent arsenite (As(III)) or pentavalent arsenate (As(V)) (Smedley and Kinniburgh 2002) (Figure 1.1). There are two main pathways through which arsenic is released into the environment: (a) natural processes (e.g. rocks' weathering, volcanic eruptions) and (b) industrial processes (e.g. mining, smelting, pesticides) (Nriagu et al. 2007). The highest concentrations of arsenic are found in groundwaters and are the result of leaching or weathering from the surrounding bedrock rock interactions (Smedley and Kinniburgh 2002).

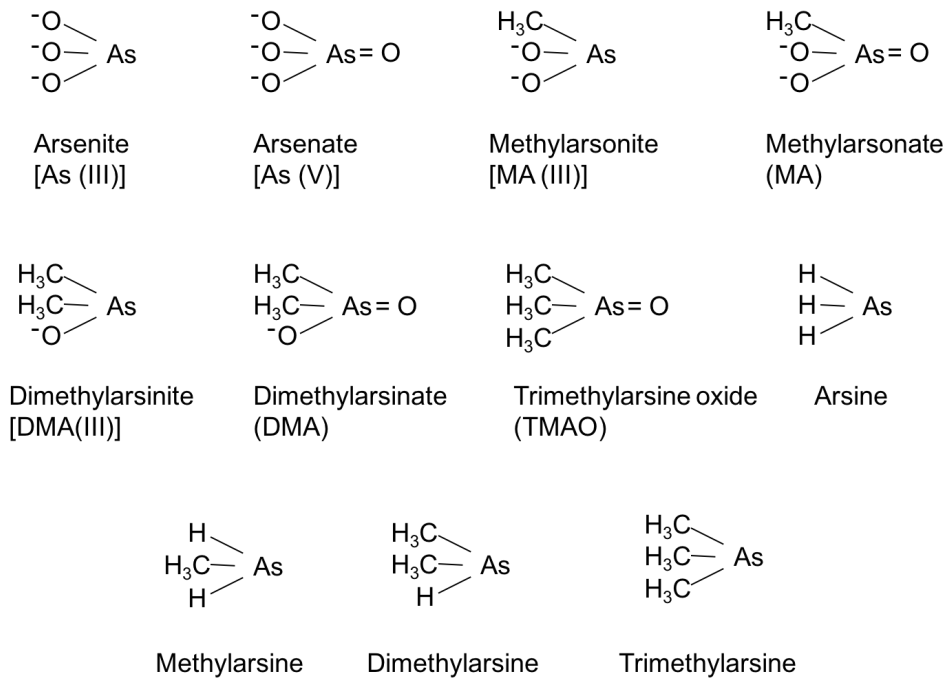

Figure 1.1. Various forms of arsenic

Arsenic has been ranked as a hazardous substance by the Agency for Toxic Substances and Disease Registry (ATSDR) and U.S. Environmental Protection Agency (EPA) since 1997 due to its toxic and carcinogenic effects on humans. Since 1958 the World Health Organisation (WHO) has been providing guidance on the recommended maximum allowance of arsenic in drinking water. In the WHO guidelines published in 1993, the guideline value of arsenic in drinking water was set to 10 µg/L ("Guidelines for Drinking-Water Quality. Third Edition. - WHO - OMS -" 2016). In the light of constantly accumulating evidence on the adverse health effects of arsenic exposure, European Union (EU) issued in June 2015 a regulation determining the maximum allowance of inorganic arsenic content in food ((EC) No 2015/1006) which was implemented on January 1st 2016.

Humans may be exposed to arsenic in several different ways. The working environment, tobacco and cosmetic products may constitute sources of arsenic exposure, however, drinking water and food are the main routes of exposure (Bundschuh et al. 2012; Fresquez, Pappas, and Watson 2013; Garrod 1999; Huq et al. 2006; Sainio et al. 2000; Sambu and Wilson 2008). Many countries worldwide have reported high levels of arsenic in their ground and surface waters. In 1962, associations were reported between arsenic in drinking water and health problems and arsenic in drinking water in Chile and in 1968 Tseng *et al.* observed a link between arsenic exposure and the prevalence of skin cancer in Taiwan (Smedley and Kinniburgh 2002; W. P. Tseng et al. 1968). The Department of Public Health Engineering in Bangladesh first detected arsenic in well-water in 1993 (British Geological Survey 1999). A study conducted in 2000 showed that the groundwater in the majority of the wells from 60 out of the 64 districts in Bangladesh contained arsenic concentrations exceeding the WHO's guideline value of 10 µg/L (Karim 2000). High levels of arsenic in drinking water have also been reported in Latin America countries, e.g. El Salvador, Nicaragua, Brazil, Bolivia, Cuba, Ecuador and Argentina (Bundschuh et al. 2012). Moreover, arsenic pollution due to industrial processes has been detected in many other countries, like U.S., Thailand, Slovakia, Turkey, China, Australia and New Zealand (Garelick et al. 2008).

More recently, human exposure to arsenic through food has been the focus of many studies. The main dietary source of exposure is rice, as it traditionally grows in water-flooded fields and can absorb more arsenic than other cereals (e.g. wheat, barley) (Sohn 2014; Williams et al. 2007). Meharg *et al.* studied the arsenic concentration in white originating from 10 different countries (Meharg et al. 2009). The study showed that the U.S. and France had the highest content of total arsenic in their rice samples, whereas the samples from Bangladesh and India contained the highest levels of inorganic arsenic (Meharg et al. 2009).

In 2015, the Swedish National Food Agency (Livsmedelverket) performed a survey on the occurrence of inorganic arsenic in rice products that were sold on the Swedish market. The survey showed that inorganic arsenic was present in the tested rice, although the concentrations did not exceed the maximum levels (0.10-0.30 mg/kg depending on the product) set by the EU (Kollander and Sundström 2015). According to the report published by the Swedish National Food Agency, arsenic exposure through food in Sweden do not pose a risk to adult health, however it poses a low-to-moderate risk for children (Sand et al. 2015).

1.2 ARSENIC METABOLISM

In order to decrease inorganic arsenic toxicity living organisms have developed detoxification mechanisms. Humans metabolize inorganic arsenic by converting it to mono- (MMA) and dimethylated arsenic (DMA), which they excrete in urine (Figure 1.2) (Marie Vahter 2002; M Vahter and Concha 2001). More specifically, the absorbed arsenate is first reduced in the blood forming arsenite, which in turn undergoes methylation in the liver (Marie Vahter 2009). The methylation, which occurs via the one-carbon metabolism, requires the presence of the enzyme arsenic(+3)-methyltransferase (AS3MT) (Marie Vahter 2009). AS3MT transfers a methyl group from S-adenosylmethionine (SAM) to arsenite in the presence of thiol-containing reductants (e.g. glutathione) (Hughes et al. 2011).

The efficiency of arsenic metabolism varies among different individuals, with efficient metabolizers excreting > 80% of total arsenic in urine in the form of DMA and poor metabolizers excreting < 60 % of total arsenic in the same form (Marie Vahter 2002). This variation in arsenic metabolism efficiency could be due to environmental, nutritional or genetic factors. For example, Kenyon *et al.* reported in 1997 that selenium (Se) could alter the metabolism of As in mice and Pilsner *et al.* suggested in 2011 that Se may reduce the body burden of As in the Bangladeshi population (Kenyon, Hughes, and Levander 1997; J. Pilsner et al. 2011). Moreover, various studies reported associations between arsenic methylation in humans and a number of enzymes, like AS3MT, N6AMT1 and DNMT1a (Engström et al. 2011; Ren et al. 2011). Evidence has been provided on polymorphisms in *AS3MT* and *N6AMT1* genes affecting the efficiency of arsenic metabolism (Engström et al. 2011; Harari et al. 2013). The first example of such polymorphisms affecting arsenic methylation came from Drobná *et al.* in 2004, who studied the ability of human hepatocytes to methylate arsenic (Drobná et al. 2004). The study revealed that hepatocytes heterozygotic for Met287Thr at amino acid base mutation of AS3MT had a higher methylation rate than the rest of the cells (Drobná et al. 2004). Later on, Schläwicke Engström *et al.* reported that three

polymorphisms in *AS3MT* introns (G12390C, C14215T, and G35991A) are associated with lower levels of MMA and higher levels of DMA in the urine of an Argentinean population (Schläwicke Engström et al. 2007).

Figure 1.2 Arsenic metabolism in humans

1.3 ARSENIC AND ADVERSE HEALTH EFFECTS

Elevated arsenic exposure has been associated with numerous adverse health effects in humans and IARC has classified arsenic as a human carcinogen. Rahman *et al.* suggested that skin can reveal initial manifestations of arsenicosis, like melanosis, keratosis and pigmentation (M. M. Rahman, Ng, and Naidu 2009). Arsenic exposure has also been shown to affect the central nervous, the renal, the urinary, the gastrointestinal and the reproductive system. Arsenic has the ability to cross the blood brain barrier and this can lead to neuropathy (Mundey et al. 2013; Vahidnia, van der Voet, and de Wolff 2007). Various studies have reported neurological effects, like peripheral neuropathy, paresthesia, memory impairment and Alzheimer's disease in relation to arsenic exposure (Vahidnia, van der Voet, and de Wolff 2007; Mukherjee et al. 2003; O'Bryant et al. 2011). Wasserman *et al.* reported in 2014 that low arsenic exposure (water As $\geq$ 5 μg/L, 70% water As $\leq$ 10 μg/L) in children in the US correlated with significant reductions in IQ scores (Wasserman et al. 2014). Since, arsenic is mainly eliminated through the renal system, this can lead to arsenic accumulation in the kidneys (Madden and Fowler 2000). Several studies have found associations between arsenic ingestion and kidney dysfunction (Wang et al. 2009; Zheng et al. 2014; Meliker et al. 2007). An increased risk for liver, renal, bladder and prostate cancer has been reported in areas with high levels of arsenic in drinking water (Liu and Waalkes 2008; Ferreccio et al. 2013; Radosavljević and Jakovljević 2008; Benbrahim-Tallaa and Waalkes 2008). Supporting data on the carcinogenicity of arsenic have also been reported based on *in vitro* arsenic exposed bladder and prostate cells (Benbrahim-Tallaa, Webber, and Waalkes 2007; Sen et al. 2007). Furthermore, arsenic ingestion has been linked to gastrointestinal symptoms, like nausea, abdominal pain and severe diarrhea (J. X. Guo et al. 2007; Ratnaike 2003).

Chronic ingestion of arsenic through drinking water has also been associated with cardiovascular and respiratory toxicity (Chang et al. 2004; Guha Mazumder 2007; Hays et al.

2008; C.-H. Tseng et al. 2003). Argos *et al.* reported in 2010 that increasing arsenic exposure through drinking water was associated with increasing mortality rate of all causes, including respiratory system defects (Argos et al. 2010). Furthermore, arsenic exposure through drinking water has been reported to increase the risk of cardiovascular disease in the United States and has also been shown to increase the pulse pressure in a study performed in Bangladesh (Y. Chen and Karagas 2013; C.-J. Chen et al. 2007). In studies from Taiwan, elevated arsenic exposure has been associated with an extreme form of vascular toxicity called Blackfoot Disease (BFD) that has been reported in Taiwan in which the blood vessels in the lower limbs get damaged by arsenic, resulting in progressive gangrene (C.-H. Tseng). Moreover, chronic exposure to arsenic through drinking water has been associated with the development of skin cancer (e.g. basal cell carcinoma, squamous cell carcinoma) in China, Bangladesh and India (H. R. Guo et al. 2001; Tondel et al. 1999; Haque et al. 2003). Several studies have also reported a link between arsenic exposure and a high mortality rate from lung cancer (Hopenhayn-Rich, Biggs, and Smith 1998; Hubaux et al. 2013).

Arsenic exposure has also been associated with adverse hematological and immunological effects. Once entering the bloodstream, arsenic binds primarily to hemoglobin and accumulates in the erythrocytes leading to hemolysis and subsequent anemia (M. Lu et al. 2004; Hall 2002). Arsenic accumulation and related epigenetic modifications have also been observed in the spleen (J. Zhang et al. 2014). Bone marrow suppression and disrupted innate immunity have also been reported in relation to inorganic arsenic exposure (Szymańska-Chabowska, Antonowicz-Juchniewicz, and Andrzejak 2002; Selgrade 2007). For example, changes in the surface markers of macrophages due to arsenic exposure can affect the cells' endocytosis and phagocytosis (Lemarie et al. 2006). In utero exposure to arsenic has also been associated with immunological defects. Ahmed *et al.* reported in 2014 that exposure to arsenic during pregnancy could affect the newborns' thymic function (Ahmed et al. 2012). Moreover, persistent arsenic exposure has been linked to decreased levels of Th1 cytokines in children (Ahmed et al. 2014).

Arsenic has been listed as an endocrine disruptor by WHO and there is constantly accumulating evidence on the adverse effects of arsenic exposure on the endocrine system. Bodwell *et al.* reported in 2004 that arsenic in very low concentrations (≤ 1 μM) can alter glucocorticoid receptor mediated induced gene expression and later Davey *et al.* suggested that arsenic can also alter retinoic acid receptor (RAR)- and thyroid hormone receptor (TR)-mediated gene expression (Bodwell, Kingsley, and Hamilton 2004; Davey et al. 2008). Furthermore, arsenic exposure has been shown to affect pancreatic activity through the

induction of pancreatic β-cell apoptosis, as well as estrogen signaling through the inhibition or induction of estrogen receptor-α expression (T.-H. Lu et al. 2011; Bae-Jump et al. 2008; J. Du et al. 2012).

1.4 ARSENIC IN CANCER TREATMENT

Apart from the adverse effects on human health, arsenic is also used in medicine. In ancient times Hippocrates used arsenic to treat ulcers, whereas in the 18th century despite the lack of therapeutic indications, doctors used as a curative agent (Waxman 2001). Nowadays, arsenic trioxide is part of the chemotherapeutic treatment against acute promyelotic leukemia (APL). The majority of APL cases is characterized by a specific chromosomal translocation, t(15;17), which fuses the PML gene to the retinoic acid receptor (RAR) α and leads to the production of the PML-RARα protein (Lavau and Dejean 1994). The fused protein acts as an aberrant RARα and has the ability to block the granulocytic differentiation (Lavau and Dejean 1994). Two studies performed by Chen *et al.* on APL cells showed that arsenic can induce apoptosis and partial differentiation on these cells (G. Q. Chen et al. 1996; G. Q. Chen et al. 1997). A combination of all-trans retinoic acid ATRA, which has been shown to also induce differentiation to APL cells, with arsenic has been recommended by the National Comprehensive Cancer Center for the treatment of relapsed APL (Lo-Coco, Cicconi, and Breccia 2015). The mean peak concentration of plasma arsenic in the individuals infused with arsenic trioxide as part of the treatment against APL may vary from 2.6 to 6.8 μM depending on the therapeutic regimen (Y. Shen et al. 2001).

Apart from APL arsenic trioxide (ATO) has also been tested as a therapeutic agent in acute nonpromyelocytic leukemia (ANPL), myelodysplastic syndrome (MDS), chronic myeloid leukemia (CML), B-cell chronic lymphocytic leukemia (CLL), hepatocellular carcinoma (HCC) and renal cell carcinoma. In the cases of ANPL, MDS and CML treatment with arsenic did not significantly improve the survival rate of the patients (Falchi et al. 2015). Zhang *et al.* suggested in 2013 that arsenic trioxide could potentially be used in the treatment of CLL, as a concentration of 2 μM ATO could induce apoptosis in B-cell chronic lymphocytic leukemia cells (X.-H. Zhang et al. 2013). Similar effects were reported from Feng-lian *et al.* in 2004, who showed that ATO (0.5-4 μM) can inhibit *in vitro* growth of renal cell carcinoma cell lines (Feng-lian et al. 2004). In 2015 Zhai *et al.* published a study showing that ATO in combination with sorafenib could inhibit the proliferation of HCC cells *in vitro* (Zhai et al. 2015).

1.5 ARSENIC-RELATED EFFECTS ON TRANSCRIPTION

The physical properties of arsenic make it a potent reactive element once entering the cell. Trivalent arsenicals have high affinity for sulfhydryl groups and can thus bind to cysteines in proteins, leading to their oxidative damage (S. Shen et al. 2013). In this respect, arsenic can influence the transcriptional processes by e.g. direct binding to transcriptional factors, via induction of oxidative stress-related signaling pathways, or via changes in the genome or the epigenome (Bustaffa et al. 2014; Ordóñez et al. 2008). Cui *et al.* reported that arsenic can downregulate the B7-H4 protein, a molecule which is upregulated on the surface of hepatocellular cancinoma cells, leading to inhibition of JAK2/STAT3 signaling (Cui et al. 2016). In human breast cancer cells 0.25-3 mM of arsenic significantly inhibited estrogen receptor mediated gene activation (Davey et al. 2007). Moreover, Hu *et al.* reported in 2002 that low dose arsenic exposure of human fibroblasts affected DNA binding activity of activating protein-1 (AP-1) and nuclear factor kappa B (NF-kB) transcription factors, both important in stress response (Hu, Jin, and Snow 2002). The study also showed that arsenic could regulate the expression of c-jun and c-fos genes, although effect on the latter was not due to the decrease in the binding activity of AP-1 and NF-kB (Hu, Jin, and Snow 2002). In 2009 Rosenblatt and Burnstein observed that ATO could inhibit the transcriptional activity of the androgen receptor (AR) via inhibiting AR binding to chromatin (Rosenblatt and Burnstein 2009). A study published by Parrish *et al.* showed that arsenic could enhance transcription factor binding to DNA, resulting in increased gene expression in renal slices (Parrish et al. 1999). Chronic arsenic exposure of renal stem cells affected Wilms tumor protein 1 (Wt-1) levels and influenced Wnt/β-catenin, Cox-2 and Bmp signaling pathways, leading to a transition to cancer phenotype (Tokar et al. 2013). Another example of ATO affecting the transcriptional activity came from Yue *et al.* in 2015, who showed that arsenic can induce the expression of Heme Oxygenase-1 (HO-1), a protein expressed in the human osteosarcoma MG63 cell line, through the translocation of nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) transcription factor from the cytoplasm to the nucleus (Yue et al. 2015). Arsenic exposure has also been shown to affect the expression of many more proteins, like CCAAT enhancer binding protein-(C/EBPs), EBV-encoded latent membrane protein-1 (LMP1) and peroxisome proliferator-activated receptor-gamma (PPAR-γ) (Yadav et al. 2013; C. Du et al.).

1.6 MASTERMIND-LIKE 1 PROTEIN (MAML1)

Mastermind-like 1 gene, is the human homolog of the *Drosophila* Mastermind gene (L Wu et al. 2000). Mastermind was first identified in *Drosophila melanogaster* as a protein whose expression can affect the neurodevelopment of the organism (Lehmann et al. 1983) and in 1995 Artavanis-Tsakonas *et al.* suggested that the mastermind gene codes for a protein which plays an important role in Notch signaling (Artavanis-Tsakonas, Matsuno, and Fortini 1995). In the beginning of the of the 21st century, the human homolog MAML1 protein was shown to be an important transcriptional co-activator in Notch signaling. In 2002 Lin *et al.* discovered two more members of the MAML family, MAML2 and MAML3, which were shown to display similar characteristics to those of MAML1 (L Wu et al. 2000; Kitagawa et al. 2001; Lin et al. 2002). Even though MAML1 does not directly bind DNA, it interacts with Notch's intracellular domain (ICN) and the transcription factor CSL forming a transcriptional activation complex, which further employs more co-activators and induces Notch-related transcription (Kitagawa et al. 2001)(Figure 1.3). Notch pathway activation occurs when the Delta– Serrate–LAG2 (DSL) ligands bind to the Notch extracellular domain (NECD) (Guruharsha, Kankel, and Artavanis-Tsakonas 2012). Following the binding, a proteolytic event takes place and the transcriptionally active Notch intracellular domain (NICD) is released into the cytoplasm (Guruharsha, Kankel, and Artavanis-Tsakonas 2012). NICD then translocates to the nucleus and interacts with MAML proteins and CSL and thereby inducing the transcription of target genes (Guruharsha, Kankel, and Artavanis-Tsakonas 2012). According to the crystal structure of the Notch transcriptional complex in humans, the ANK domain of Notch and the transcription factor CSL form a binding pocket, which interacts with the N terminus of MAML1 (Choi et al. 2012). MAML1 protein also recruits p300/CBP co-activator proteins to the Notch transcriptional complex and this interaction is critical for the initiation of transcription (Fryer et al. 2002). On the other hand, the interaction of MAML1 with the cyclin-dependent kinase (CDK) 8 has been shown to play a role in Notch transcriptional complex degradation (Fryer, White, and Jones 2004).

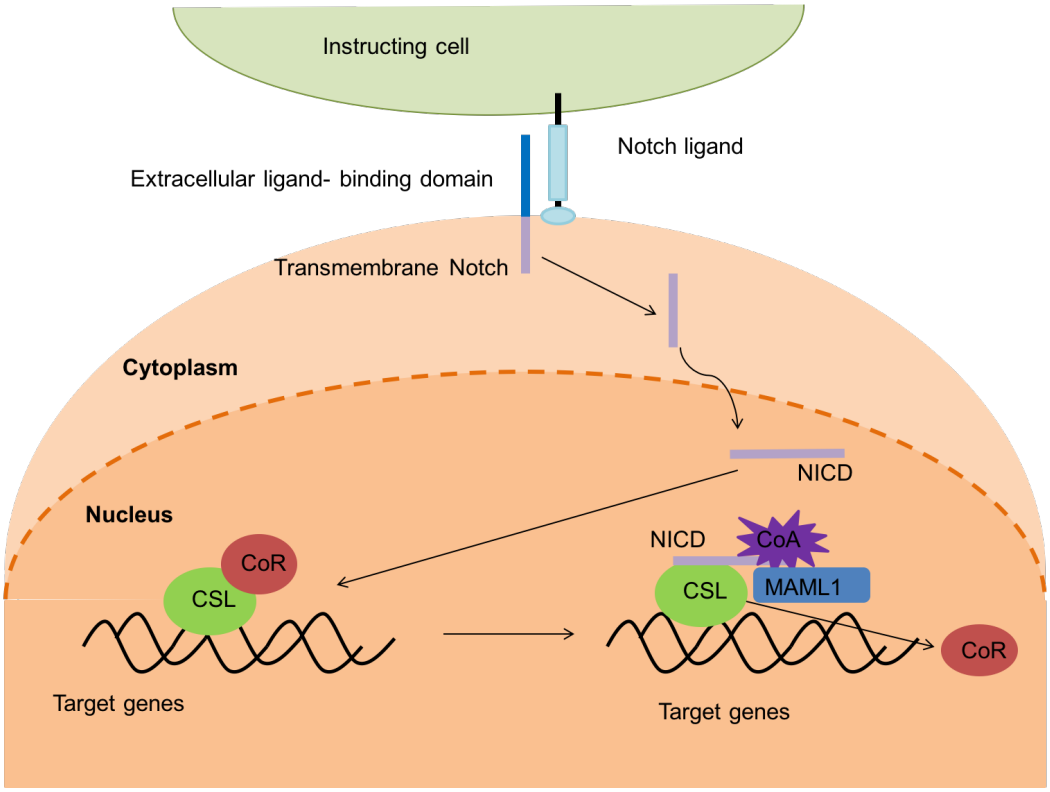

Figure 1.3 Description of Notch signaling pathway

Other studies performed on MAML1 showed that apart from Notch signaling, MAML1 also acts as a co-activator in various other pathways. In 2006, Shen *et al.* suggested that MAML1 is involved in myogenesis independently of the Notch signaling pathway as it had previously been shown that activation of the Notch signaling pathway inhibits myoblast differentiation (H. Shen et al. 2006; Kopan, Nye, and Weintraub 1994). McElhinny *et al.* observed that MAML1 can interact with the muscle-specific transcription factors MEF2C and myogenin and thus induce myogenic differentiation (McElhinny, Li, and Wu 2008). Notch signaling activation blocks MAML1-induced differentiation and leads to the recruitment of MAML1 to the Notch transcriptional complex (McElhinny, Li, and Wu 2008). Moreover, MAML1 has been reported to co-activate p53, which is a transcription factor involved in many different pathways, like developmental, stress-response and apoptotic (Vousden and Lane 2007). Zhao *et al.* showed in 2007 that MAML1 is part of the p53 activator complex recruited on the target genes and that the N-terminus of MAML1 interacts with the DNA binding domain of p53 (Zhao et al. 2007). Apart from p53, MAML1 is also involved in the Wnt/β-catenin pathway (Figure 1.4), which regulates various cellular processes, like cell fate determination, organogenesis and stem cell renewal (Komiya and Habas 2008). In the absence of a Wnt

signal, the cytoplasmic β-catenin is continuously degraded by the Axis complex (MacDonald, Tamai, and He 2009). Once a Wnt ligand binds to the Frizzled (Fz) receptor, which is a transmembrane protein, and its co-receptor LRP6 protein, the Axis complex is recruited to the

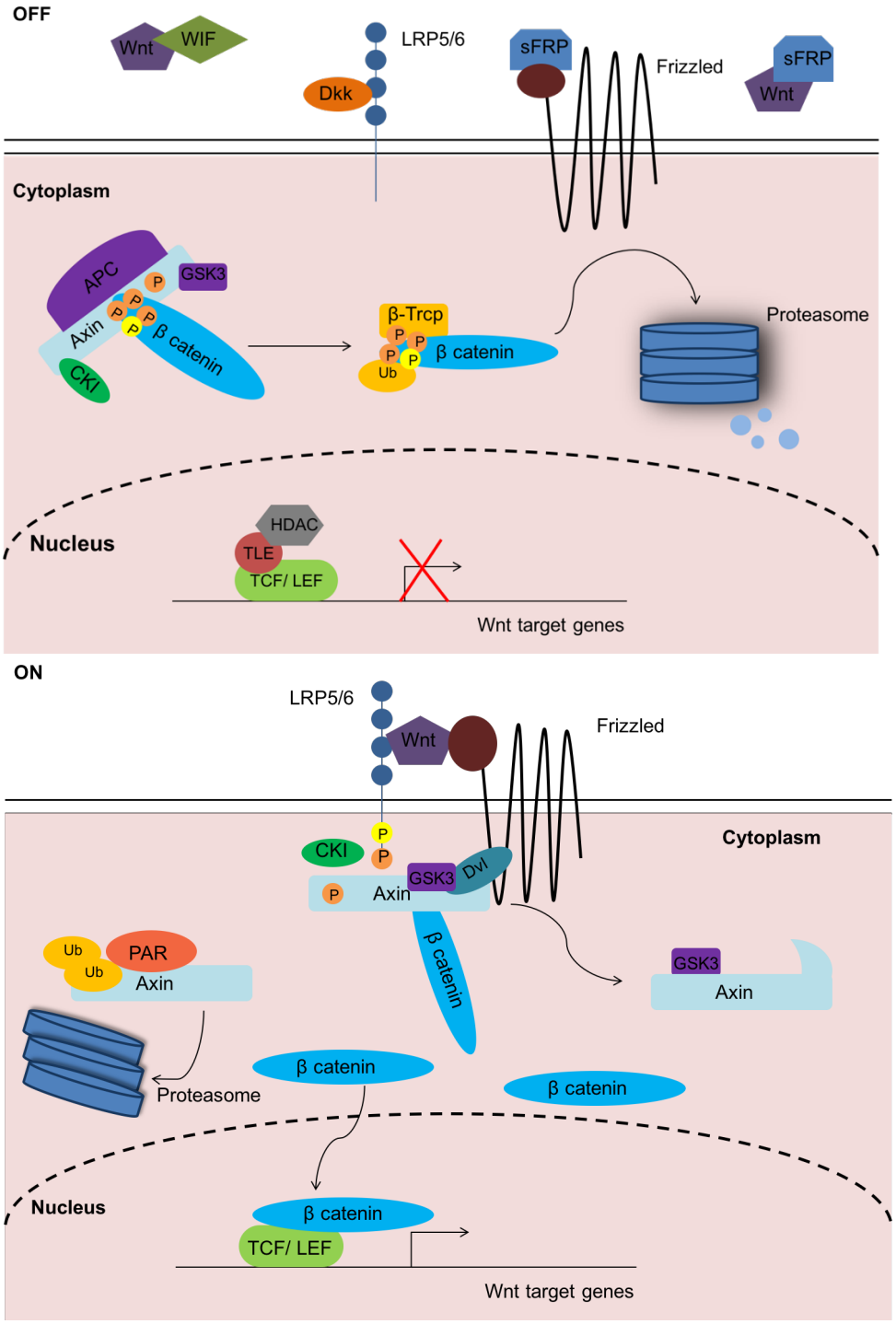

Figure 1.4 Description of Wnt/βcatenin signaling

Wnt-Fz- LRP6 complex (MacDonald, Tamai, and He 2009). In this way the Axis complex does not induce degradation of β-catenin and the latter can enter the nucleus and activate Wnt target gene transcription through its binding to the TCF/LEF transcription factors (MacDonald, Tamai, and He 2009). According to Alves-Guerra *et al.* MAML1 is recruited by β-catenin on the *cyclin D* and *c-Myc* promoters, both targets of the Wnt signaling pathway, and it affects β-catenin/TCF activity in colon carcinoma cells (Alves-Guerra, Ronchini, and Capobianco 2007).

1.7 MAML1 IN DISEASE

Since MAML1 plays a significant role in many signaling pathways, changes in the expression or the structure of the protein can have adverse effects on various processes. As a result of the involvement of MAML1 in Notch signaling, deletions of this protein have been shown to affect marginal zone B-cell development and T-cell differentiation (Maillard et al. 2004; Lizi Wu et al. 2007). Watanabe *et al.* observed that MAML1 enhances the activity of Runx2 transcriptional factor affecting in this way chondrocyte maturation during bone development (Watanabe et al. 2013). Moreover, a study published in 2007 by Wu *et al.* showed that the expression pattern of MAML1 differed among the various tissues in mouse embryos, suggesting a tissue-specific involvement of MAML1 in cell fate and differentiation (Lizi Wu et al. 2004).

Many studies have focused on the involvement of MAML1 protein in tumor development and therapeutical approaches. Proweller *et al.* showed that inhibition of Notch signaling through the use of dominant-negative MAML1 can lead to the development of squamous cell carcinoma in mice and Alves-Guerra *et al.* reported that knockdown of MAML1 could decrease colon carcinoma cell survival (Proweller et al. 2006; Alves-Guerra, Ronchini, and Capobianco 2007). Also, knockdown of MAML1 in B16 melanoma cells can lead to changes in the tumor microenvironment by the secretion of chemokines and cytokines and can also induce cell senescence and differentiation (Kang et al. 2013). Forghanifard *et al.* published a study in 2012 showing that increased MAML1 expression is associated with lymph node metastasis in patients with esophageal squamous cell carcinoma and suggested that MAML1 could be used as a molecular marker of tumor progression (Forghanifard et al. 2012). Furthermore, Hansson *et al.* suggested that MAML1 enhances EGR1 transcription factor activity and may play a role in renal cancer cell carcinoma and in 2015 Feng *et al.* reported that *MAML1* and *KAT2B* copy number variances were predominant changes in patients with clear-cell renal cell carcinoma (ccRCC) (Hansson et al. 2012; Feng et al. 2015).

MAML1 has also been reported to suppress cervical cancer cell viability via its involvement in the NF-kB pathway (Kuncharin et al. 2011).

1.8 EPIGENETIC REGULATION

Epigenetics refers to stable, heritable or long-term non heritable alterations in the potential of gene expression, distinct from DNA sequences, that take place during development or cell proliferation (Margueron and Reinberg 2010). The main mechanisms involved in epigenetic regulation are DNA methylation, non-coding RNAs and histone modifications (Margueron and Reinberg 2010) (Figure 1.5). In mammals, DNA methylation occurs in cytosine–phosphate-guanine (CpG) regions at the fifth carbon of cytosine and is the result of the activity of three conserved enzymes, DNA methyltransferase 1 (DNMT1), DNMT23A and DNMT3B (Margueron and Reinberg 2010). Even though it may be the combination of the epigenetic markers that defines transcriptionally active regions, hypermethylation in CG-dense regions (CpG islands) has been linked to transcriptional silencing (Smith and Meissner 2013). Changes in the methylation pattern of specific gene promoters has been related to many malignancies, e.g. *BRCA1* in breast cancer, *VHL* in renal cancer and *MLH1* in colorectal cancer (Baylin 2005; Jones and Baylin 2002).

Non-coding RNAs are divided in two big groups, small non-coding RNAs (< 200 nucleotides) and long non-coding RNAs (>200 nucleotides), and have lately been shown to play an important role in gene regulation and chromatin remodeling in the mammalian genome (Cao 2014; Costa 2008). The affinity of these anti-sense RNAs to specific sequences in the genome and their ability to recruit chromatin modifiers enables non-coding RNAs to establish chromatin modification (e.g. DNA methylation, histone modification) in targeted regions and thus regulate gene expression (Costa 2008). Disruption of non-coding RNAs activity has been linked to a series of diseases. For example, changes in miR-124a micro RNA have been related to colon and gastric cancer, whereas changes in miR-205 have been associated with bladder cancer (Esteller 2011). Moreover, non-coding RNAs alterations are involved in neurological disorders, like Alzheimer's and Parkinson's disease (Esteller 2011; Hébert et al. 2010; Kim et al. 2007).

Post-translational histone modifications (PTHMs) have been extensively studied. Histones' N-terminal tails can protrude from the nucleosome and be the substrate for post-translational modifications, like acetylation, methylation and phosphorylation (Bannister and Kouzarides 2011). Histone acetylation is regulated by the activities of two types of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Bannister and

Kouzarides 2011). HATs are divided in three categories, general control non-derepressible 5 (Gcn5)-related N-acetyltransferases (GNATs), p300/CBP and MYST proteins, and in the presence of acetyl-CoA they can transfer an acetyl group to the histone lysine side chains (X.-J. Yang and Seto 2007). HDACs remove the acetyl group from the lysines and can be divided in three groups; class I which includes RPD3-like proteins, class II which includes HDA1-like proteins, and class III which includes only maize HD2 protein (Cress and Seto 2000). Eight different HDACs exist in humans (HDAC1-8), which all belong to the first two classes (Cress and Seto 2000). Regarding histone methylation, this occurs on the side chains of arginines and lysines and is regulated by several histone methylases and demethylases (Bannister and Kouzarides 2011). Histone methylases can transfer methyl groups from S-adenosylmethionine (SAM) to the histone tails (Bannister and Kouzarides 2011). Many more histone modifications have been reported, like deimination, β-N-acetylglucosamine, ADP ribosylation, ubiquitylation, sumoylation, histone tail clipping and histone proline isomerization (Bannister and Kouzarides 2011).

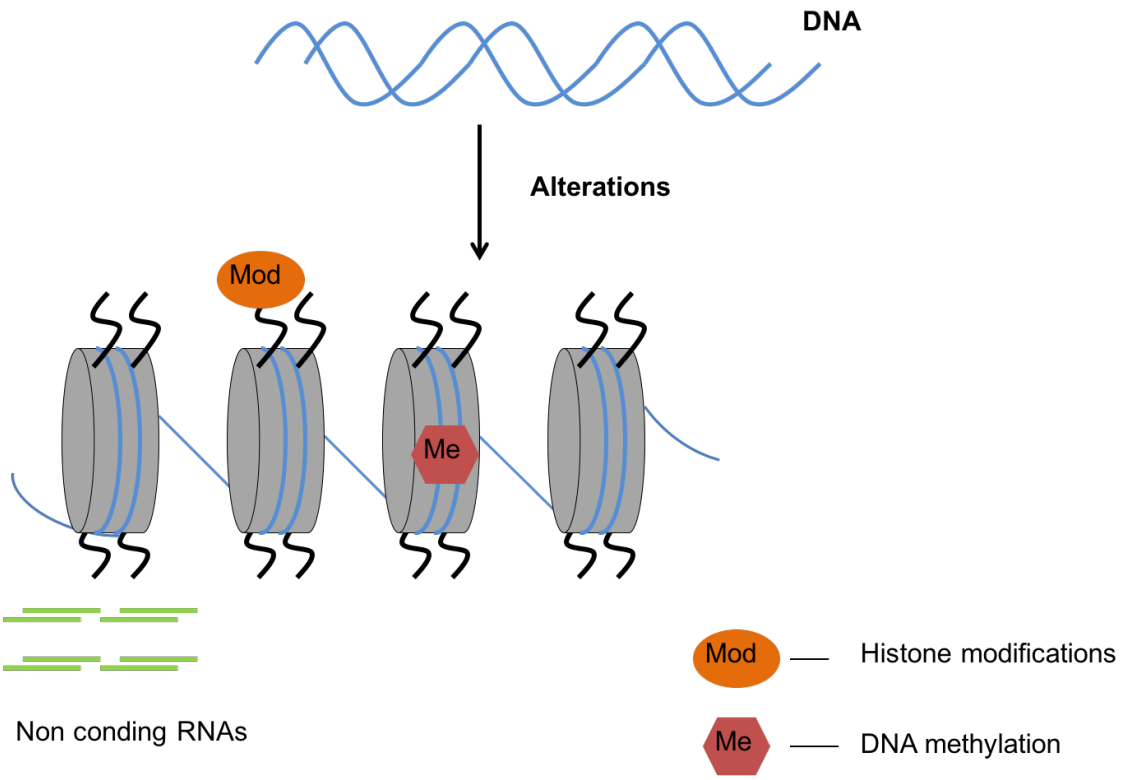

Figure 1.5 Epigenetic regulation mechanisms

Several studies have focused on the role of histone methylation and acetylation in gene expression. Generally, in mammals histone 3 lysine 9 acetylation (H3K9Ac) and H3K4 methylation are linked to active gene expression, whereas H3K9, H3K27 and H4K20 methylation are associated with transcriptional repression (Wiencke et al. 2008). On the other hand, Barski *et al.* reported in 2007 that methylation of histone 3 lysine 7 (H3K27) and H3K9 can activate gene transcription in CD4+ T cells, whereas trimethylation of the same residues leads to gene inactivation (Barski et al. 2007). This last observation suggests that the same histone modification may either induce or repress transcription depending on the context. Vakoc *et al.* also reported a similar finding with H3K9me3 being present in transcriptionally active genes (Vakoc et al. 2006).

Various complexes have been identified, which link transcriptional activation to the establishment of the epigenetic marks. Robertson *et al.* observed in 2000 that DNMT1 interacts with the retinoblastoma (Rb) tumour suppressor gene product, the transcription factor E2F1 and HDAC1 forming a complex which leads to transcriptional repression of E2F target genes in HeLa cells (Robertson et al. 2000). Brown *et al.* also reported interaction between the histone acetyltransferases SAGA and NuA4 and acidic transcriptional activators (Brown et al. 2001). Another link between epigenetic regulation and gene expression is the DNMT1-PCNA-HDAC2 complex, which combines DNA methylation with histone deacetylation in order to establish transcriptional repression in replication foci (Rountree, Bachman, and Baylin 2000).

1.9 EFFECT OF ARSENIC ON EPIGENETIC REGULATION

Apart from leading to genetic instability through the induction of genotoxic damage to the cells, arsenic exposure can also affect the epigenome (Bustaffa et al. 2014). In 2012 Du *et al.* observed DNA hypomethylation in the promoter of estrogen receptor α (*ESR1*) and a subsequent re-expression of this previously silenced receptor in arsenic exposed breast cancer cells (J. Du et al. 2012). Moreover, Mass and Wang observed that arsenic induced hypermethylation of the *TP53* promoter in lung adenocarcinoma cells and Chanda *et al.* observed the same effect in blood samples from individuals chronically exposed to arsenic (Mass and Wang 1997; Chanda et al. 2013; Broberg et al. 2014; Hossain et al. 2012). Changes in global methylation levels have also been reported in relation to chronic arsenic exposure. Niedzwiecki *et al.* observed a positive correlation between arsenic exposure and global DNA methylation levels in peripheral blood mononuclear cells in Bangladeshi adults (Niedzwiecki et al. 2013). In 2007 Pilsner *et al.* suggested that folate could influence arsenic-

induced changes in the global methylation profile of peripheral blood leukocytes from Bangladeshi adults (J. R. Pilsner et al. 2007). Even though the effect of arsenic on non-coding RNAs has only lately been studied, there is accumulating evidence that such an exposure can deregulate microRNAs. For example, Ling *et al.* observed an increase in miR-21 levels in relation to chronic arsenite exposure in human embryo lung fibroblast cells (Ling et al. 2012). Moreover, Sturchio *et al.* reported a list of miRNAs, including miR-663, miR-638 and miR-150, whose expression is affected by exposure to arsenic (Sturchio et al. 2014).

Apart from DNA methylation and non-coding RNAs, exposure to arsenic also influences histone modifications as well. Jensen *et al.* reported that arsenic could induce malignant transformation in bladder cells through changes in H3 acetylation of a number of genes (Jensen et al. 2008) and Tyler *et al.* observed changes in H3K9Ac and H3K4me3 levels in the brains of adult mice as a result of perinatal arsenic exposure (Tyler et al. 2015). Cronican *et al.* also observed hypoacetylation in H3K9 in adult mice brains in relation to prenatal arsenic exposure (Cronican et al. 2013). Moreover, Rahman *et al.* reported an increase in H3K9Ac global levels mediated by an imbalance in HDAC2 and PCAF, an acetyltransferase, levels in relation to arsenic exposure in embryonic kidney (HEK) 293T cells (S. Rahman et al. 2015). Zhou *et al.* reported in 2008 that exposure to arsenite increased H3K9me2 and decreased H3K27me3 levels in lung carcinoma A549 cells (Zhou et al. 2008). The study also showed a correlation between the changes in global H3K9me2 levels and an increase in G9a levels, a histone methyltransferase, levels (Zhou et al. 2008). Furthermore, Chervona *et al.* observed correlations between arsenic exposure and PTHMs in peripheral blood mononuclear cells coming from a population-based study in Bangladesh (Chervona et al. 2012). In this study positive correlations were reported between urinary arsenic and H3K9me2 and inverse correlations were reported between urinary arsenic and H3K9Ac (Chervona et al. 2012). A sex-specific pattern was also observed for H3K27me3 and H3K4me3, which correlated positively with water arsenic in females and inversely in males (Chervona et al. 2012).

1.10 CONCLUSION

In conclusion, the existing data suggest that arsenic has multiple effects on the epigenetic and transcriptional regulation of the cells. However, further studies are needed in order to acquire a deeper understanding on arsenic's mode of action in the intracellular environment.

2 AIMS OF THE THESIS

Despite the fact that arsenic related toxicity and carcinogenicity has been the subject of many studies throughout the years, the exact mechanisms via which arsenic affects human cells have not been completely elucidated. In this thesis, we aimed to investigate the effects of arsenic on transcriptional and epigenetic regulation in both normal cells and cancer cells. More specifically, we aimed to:

- Elucidate the mechanisms that regulate MAML1 protein levels, by investigating MAML1 ubiquitination, by mapping the lysines in MAML1 that are targets of ubiquitination and identifying MAML1 interacting coregulators affecting MAML1 ubiquitination. (**Paper I**).
- Investigate whether MAML1 is involved in kidney cell proliferation and effects of arsenic, since MAML1 is presumed to be involved in renal cancer development (**Paper II**).
- Investigate MAML1 involvement in epigenetic regulation via a direct interaction with the repressive complex DNMT1-PCNA-HDAC2 and to elucidate whether the members of the complex are affected by arsenic exposure (**Paper II-III**).
- Investigate whether arsenic exposure affects the global levels of H3K9me3 and H3K9Ac, both histone modifications that are involved in transcriptional repression and activation respectively, in T lymphocytes *in vivo* and *in vitro* (**Paper III**).

3 MATERIALS AND METHODS

This section is a summary of the materials and methods used in this thesis. For further details, the reader is referred to the individual papers. (**Paper I-III**)

3.1 PLASMIDS

In order to study MAML1 ubiquitination in **Paper I**, human–MAML1, MAML2, MAML3, CBF1 and deletion mutants of MAML1 (1–939, 1–710, 1–579, 1–478 and 1–301) were cloned into pCS2. We generated MAML1 K/R (K112, 178, 188, 189, 405, 407, 639, and 822) mutant by using site-directed mutagenesis. Both MAML1 and MAML1 K/R were sub-cloned into a p3X-FLAG vector. Heme-agglutinin tagged ubiquitin (HA-Ub) expression plasmid, pCI-FLAG-p300 and pCI-FLAG-p300ΔHAT were also used.

To create the HEK293-MAML1 cell line used in **Paper III** pCDNA3.1-FLAG-MAML1 (1–1016) plasmid was used.

3.2 CELL LINES AND CONSTRUCTS

The CD4+ T-lymphocyte cell lines (Jurkat and CCRF-CEM) were used as well as the human embryonic kidney cell line (HEK293) and a cervical adenocarcinoma cell line (HeLa) Moreover, a HEK293-MAML1 cell line was created, in which MAML1 protein was overexpressed. (**Paper I-III**)

3.3 REPORTER GENE ASSAYS

In order to detect the effects of MAML1 constructs and their interaction with NICD, CDK8 and p300 on the Notch signaling target HES-1 promoter, we performed three reporter gene assays (**Paper I**). In the first one HeLa cells were transiently transfected with HES1-Luc reporter, pCS2-N1ICD, pCS2-MAML1 and pRL-TK and in the second one with HES1-Luc reporter, pCS2-Notch1 ICD, FLAG-MAML1 or FLAG-MAML1K/R and pRL-TK. We harvested the cells after 40–48 h and the the Dual Luciferase Assay System from Promega was used in order to measure levels of luciferase. In the third assay, we cotransfected HeLa cells with pG5-luc reporter and GAL4-N1 ICD, p300-HA, CDK8-FLAG and MAML1 plasmids and 48 h later the cells were harvested and LucySoft3 (Anthos Labtec, Salzburg, Austria) was used in order to measure luciferase activity.

3.4 IMMUNOSTAINING

In **Paper I** we seeded HeLa cells into a Lab Tek II 8 well chamber slide system (ThermoScientific) in order to investigate the intracellular localization of the MAML1 and MAML1 K/R proteins. The cells were incubated for 24 hours and transfection with MAML1 WT or MAML1 K/R expression plasmid followed. Fixation and permealization of the cells as well as blocking of the slides followed. The cells were incubated with anti-FLAG epitope tag primary antibody and later on with goat anti-mouse FITC conjugated secondary antibody. After washing the cells, we proceeded with Hoechst 33258 staining (Invitrogen). Finally, the cells were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA) and fluorescence microscopy was used for cellular imaging.

In order to investigate the localization of MAML1, HDAC2 and PCNA in HEK293 cells in **Paper III**, we grew HEK293-MAML1 cells on glass slides for 48 hours and then washed, fixed and permeabilized them. Following washing and blocking of the slides, the cells were immunostained with primary antibodies against MAML1, HDAC2 and PCNA. Subsequently, the cells were washed and incubated with secondary antibodies and thereafter one hour of incubation staining with DAPI was performed. After another round of washing, the slides were mounted and analyzed by fluorescence microscopy.

3.5 *IN VITRO* TRANSCRIPTION ASSAY

In **Paper I** we performed an *in vitro* transcription assay in order to investigate functional interactions among MAML1, Notch, p300 and CDK8. We used purified recombinant Drosophila Acf-1, ISWI, and NAP1 proteins and assembled a chromatin molecule containing 12 binding sites for CSL. In order to induce transcription, the chromatin template was first incubated with N1ICD, CSL, MAML1, p300, CDK8 and acetyl-CoA and then HeLa nuclear extract and NTPs were added. Reverse transcription in the presence of 32P-labeled probes followed and the products were analyzed on polyacrylamide gels. Quantification was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

3.6 ANALYSIS OF MUTATIONS IN MAML1

In **Paper I** we searched into the Catalogue of Somatic Mutations in Cancer (COSMIC) database in order to investigate whether MAML1 mutations exist in various cancer cell lines (Forbes et al. 2014).

3.7 UBIQUITINATION EXPERIMENTS

In order to detect MAML ubiquitination in **Paper II** we performed immunoprecipitation experiments. In the beginning, we transfected HeLa cells with myc-tagged human mastermind constructs (MAML1-3) together with HA-Ub. Following a 24 h incubation, we lysed the cells and sonicated the lysates in order to remove the cell debris. After pre-clearing the supernatants with Protein G PLUS beads (Santa Cruz Biotechnology) and centrifuging the lysates, anti-myc (9E10) antibody was added and overnight incubation at 4°C followed. The next day immunoprecipitation (IP) was performed and the protein samples were analyzed with SDS-PAGE.

3.8 PULSE CHASE EXPERIMENTS

In order to determine the half-lives of MAML1-3 in **Paper II** we performed a series of pulse chase experiments. We performed lipofectamine transfections for the different Myc-tagged MAML constructs following the procedures mentioned above. After a 24 h incubation time, we treated the cells with cycloheximide and collected cell extracts every hour for the next 5 hours. We processed the samples as mentioned in **§3.7**, without though performing IP. The samples were analyzed with SDS-PAGE.

3.9 SDS-PAGE AND WESTERN BLOT ANALYSIS

Protein samples were analyzed with SDS-PAGE on acrylamide gels of various concentrations (**Paper I-III**). The proteins were transferred on PVDF membranes and blocked in room temperature for 1 hour. Following, the membranes were incubated with primary antibodies against Myc, FLAG, GAPDH, MAML1, PCNA, DNMT1, HDAC2, H3K9me3, H3K9Ac and beta-actin overnight. The next day secondary antibodies were added and developing followed by using ECL solutions (GE Healthcare). In order to quantify protein concentrations coming from the Western blot experiments we used ImageJ software (NIH, Bethesda, MD, USA).

3.10 CELL PROLIFERATION ASSAY

In order to assess cell proliferation in HEK293 and HEK293–MAML1 cells (**Paper II**) we performed MTS assay. In short, cells were seeded in 96-well plates and cell viability was assessed every 24 hours for 3 days by spectrophotometry following the manufacturer's instructions. Concerning the siRNA experiments, the MTS assay was performed 24 hours after transfecting HEK293 cells with MAML1 siRNA following the process described above.

3.11 CELL CULTURE ASSAY

In order to investigate the effects of arsenic on MAML1 in **Paper II**, we seeded HEK293 cells in 6-well plates and treated them with 1μM and 5 μM of sodium meta-arsenite (AsNaO2). After 48 hours incubation, we performed cell lysis and the cell extracts were prepared for SDS-PAGE.

In **Paper III** we performed cell culture assays in order to investigate the effects of arsenic on T lymphocytes. Jurkat and CCRF-CEM cells were seeded in 6-well plates and treated with 0.1 μg/L, 1 μg/L and 100 μg/L of sodium meta-arsenite (AsNaO2). Cell lysis was performed after 48 and 72 hours of incubation and the extracts were prepared for SDS-PAGE as described above.

3.12 RNA EXTRACTION

In order to investigate any effects of arsenic on the MAML1 expression we performed RNA extraction (**Paper II**). We seeded HEK293 cells in 6-well plates, treated them with 1 μM and 5 μM of sodium meta-arsenite ($AsNaO_2$) and following 24 hours of incubation we extracted RNA using a RNeasy Mini Kit (Qiagen).

3.13 REAL-TIME PCR

We performed real-time PCR in order to investigate the effects of arsenic on MAML1 mRNA levels (**Paper II**). In brief, we performed cDNA synthesis and mRNA levels were detected using MAML1 specific forward and reverse primers. The data were normalized by using mRNA levels of the 36B4 (RPLP0) housekeeping.

3.14 FLOW CYTOMETRIC DETERMINATION OF APOPTOSIS

Flow cytometry was performed in **Paper II** in order to determine apoptosis in arsenic treated HEK293 cells. In short, treated HEK293 cells were stained with annexin V-FITC and propidium iodide (PI) using Oncogene Research Products detection kit and the analysis was performed on a FACScan. Apoptosis was defined as presence of annexin V-positive/PI-negative cells, whereas necrosis was defined as the detection of positive for both annexin V and PI cells.

3.15 MEASUREMENT OF TOTAL ARSENIC AND ARSENIC METABOLITES

In **Paper II** we investigated the possible presence of different arsenic species in HEK293 cultures. In brief, we seeded HEK293 cells in 6-well plates and treated them with 0.1, 1 or 5 μM of sodium meta-arsenite ($AsNaO_2$). After 48 and 96 hours of incubation cell lysates and debris were obtained as described in **§3.13**. Total arsenic was detected in cell media, lysates, and debris after microwave digestion using an Agilent 7700x ORS ICP-MS (Agilent Technologies, Tokyo, Japan). Separation and detection of inorganic arsenic metabolites was performed on Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with hydride generation (HG) ICP-MS (Agilent 7500ce, Agilent Technologies, Tokyo, Japan).

3.16 INVESTIGATION OF *MAML1* CORRELATIONS WITH SELECTED GENES IN TRANSCRIPTOMICS DATABASES AND DATASETS IN THE PUBLIC DOMAIN

In order to investigate possible correlations of *MAML1* with *DNMT1*, *PCNA*, *CDK2*, *XRCC5* (*Ku80*) and *HDAC2* genes (**Paper II-III**), we used the public In Silico Transcriptomics (IST) database and an Array Express independent microarray dataset, containing gene expression data from normal and diseased tissues. The statistical analysis of the data was performed as mentioned in **§3.11**.

3.17 GST-PULL-DOWN ASSAY

In order to identify MAML1 protein interactions with HDAC2, PCNA, DNMT1 and CDK8 we performed a GST-pull-down assay (**Paper III**). In brief, following the expression and purification of Glutathione S-transferase (GST)-tagged MAML1 in the *Escherichia coli* strain BL21, glutathione-Sepharose beads with bound GST-MAML1 were incubated with HEK293 whole-cell extract. After washing steps, the isolated proteins were analyzed by SDS-PAGE and immunoblot.

3.18 CO-IMMUNOPRECIPITATION

To investigate MAML1 protein interactions with HDAC2, PCNA, DNMT1 and CDK8 co-immunoprecipitation was performed (**Paper III**). During this assay, MAML1 protein was immunoprecipitated from HEK293-MAML1 whole cell extract and we analyzed both the input and the IP samples using immunoblot.

3.19 LYMPHOCYTES ISOLATED FROM ARSENIC EXPOSED INDIVIDUALS

In **Paper III,** we explored if the levels of H3K9me3 and H3K9Ac in CD4+ and CD8+ cells isolated from arsenic-exposed individuals were associated with their urinary arsenic concentrations. These individuals, in total 28 women, are residing in San Antonio de los Cobres and surrounding villages at around 4,000 m above sea level in the Andes Mountains, Salta Province, Argentina. In this study area, the inhabitants are exposed to varying concentrations of arsenic via their drinking water. At the recruitment in 2011, the women were interviewed and both a blood and spot urine sample was collected. As indicated, above, the blood samples were sorted for CD4+ and CD8+ cells (Dynabeads kit, Life Technologies, CA, USA) immediately after blood collection. The women´s arsenic exposure was assessed based on the concentration of metabolites of inorganic arsenic in their urine **§3.17**. Ethical permission for the present study was obtained from the Stockholm Regional Ethical Review Board as well as the Ministry of Health in Salta, Argentina (2008/1430-31). A more detailed description is given in **Paper III**.

3.20 STATISTICAL ANALYSES

In **Paper I and III**, differences between the control and treated samples were tested using the two-sided student's t-test. In **Paper II** and **III,** Pearson's correlation was used for the analysis of the data derived from the transcriptomics databases. In **Paper III,** Mann-Whitney U test was used test whether the histone modifications' levels differed between individuals with a high or low arsenic exposure (defined as median value of urinary arsenic). For all the tests described above a p-value < 0.05 were considered as statistically significant.

4 RESULTS AND DISCUSSION

4.1 UBIQUITINATION OF MAML PROTEINS

MAML proteins are transcriptional co-activators of numerous transcriptional factors and have been shown to be involved in various signaling pathways, including Notch and β-catenin (Lin et al. 2002; McElhinny, Li, and Wu 2008). Disruption of MAML expression or activity has been linked to deregulation of tissues' normal development and tumorigenesis (Watanabe et al. 2013; Proweller et al. 2006; Kang et al. 2013). Maillard *et al.* observed in 2004 that MAML1 is critical for T cell development, via regulating Notch1 signaling, and in 2007 Wu *et al.* showed that MAML1 was required for marginal zone B-cell development, via influencing the Notch2 signaling pathway (Maillard et al. 2004; Lizi Wu et al. 2007). Moreover, Notch signaling deregulation by MAML proteins has been linked to tumor development, including cutaneous melanoma, neuroblastoma and renal cancer (W. Zhang et al. 2015; Heynen et al. 2016; Hansson et al. 2012). In this respect and considering that ubiquitination can lead to degradation of transcriptional activators' and co-activators' and thus shutting down of transcriptional processes, we investigated in **Paper I** whether the proteins of the MAML family (MAML1-3) are subjected to ubiquitination and how that would affect Notch signaling (Geng, Wenzel, and Tansey 2012).

We initially verified that MAML1 is an important part of Notch signaling in our cell system by overexpressing it together with N1ICD and we observed that MAML1 increased the activation of HES1 promoter, which is a known Notch target. We also reported that the C-terminus of MAML1 plays an important role in its regulatory activity in Notch signaling, since MAML1 lacking the C-terminus had decreased activation of the HES1 promoter.

In the next step we peformed ubiquitination experiments on the MAML proteins. We reported that MAML1 protein is ubiquitinated, which subsequently decreases the protein's half-life. We also identified 8 lysine residues (K112, 178, 188, 189, 405, 407, 639, 822) which played a major role in this process, since mutating all of them at the same time decreased the MAML1 ubiquitination by 95 %. These lysine residues were mapped in the region 75-300 of the MAML1 protein, which has previously been shown to bind protein p300 (Saint Just Ribeiro, Hansson, and Wallberg 2007), and their simultaneous mutation to arginine led to decreased HES1 promoter activation. We thus suggested that disruption of MAML1 ubiquitination via the mutation of these lysine residues affects Notch signaling.

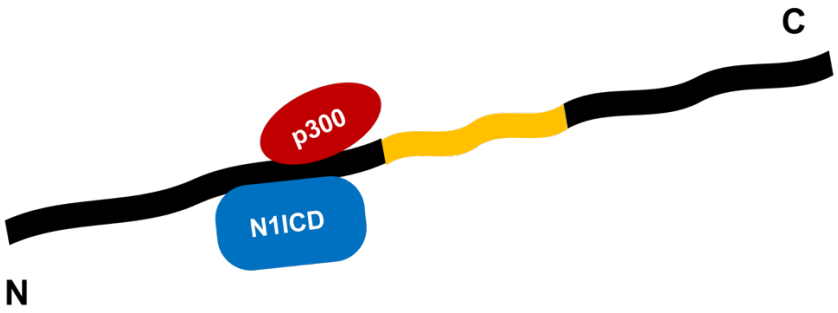

Figure 4.1 MAML1 ubiquitination takes place in the region (yellow) close to p300 and N1ICD binding sites

While investigating how MAML1 ubiquitination is regulated, we observed that it is affected by p300 and N1ICD, both binding partners of MAML1. More specifically, overexpression of p300 together with MAML1 stimulated MAML1 ubiquitination and decreased its half-life, whereas deletion of the p300 binding region on MAML1 (75-300 aa) stabilized the half-life of MAML1. Since the deletion of the p300 binding region in MAML1 still leaves 4 lysine residues that can still be involved in ubiquitination, but the MAML1 300-1016 protein is only weakly ubiquitinated, we speculated that p300 may assist in the recruitment of a ubiquitin ligase on MAML1. We further speculate that the deletion of the p300 binding region on MAML1 does not enable the ubiquitin ligase's recruitment. Another interesting observation was that p300 does not need its histone acetylase (HAT) activity in order to induce MAML1 ubiquitination. It has previously been reported that p300 mediates MAML1 acetylation, something that could decrease p300-MAML1 interaction and lead to reduced transcriptional activity (Saint Just Ribeiro, Hansson, and Wallberg 2007). It would thus be expected that MAML1 acetylation would also lead to an increase in the protein's ubiquitination, but as we reported in **Paper I** loss of p300 HAT activity did not affect MAML1 ubiquitination. Further studies are needed in order to determine what is the relation between these two modifications.

On the other hand, we observed that overexpression of N1ICD together with MAML1 decreased the levels of MAML1 ubiquitination. It has previously been shown that N1ICD binds to the first 75 aa of MAML1 (Kitagawa et al. 2001; L Wu et al. 2000), adjacent to the region that is important for MAML1 ubiquitination. We thus suggested that N1ICD binding may inhibit ubiquitin ligase's interaction with MAML1 and thus stabilize MAML1.

Concerning MAML2 and MAML3 ubiquitination, we tested two cell lines, HEK293 and HeLa, and in both cases no ubiquitination was detected. However, we observed differences in the half-lives of MAML2 and MAML3 in comparison to MAML1, with the latter being statistically significant. To clarify whether this difference in the half-lives of MAML2 and MAML3 compared to MAML1 could be due to different number of residues being ubiquitinated, we run a CLUSTALW alignment of MAML1-3. The results suggested that the lysine residues detected in MAML1 were not conserved in MAML2 or MAML3, except for K162 in MAML2 (K112 in MAML1) and K190 in MAML3 (K178 in MAML1). Considering all of the arguments above we suggested that MAML2 and MAML3 regulation may differ compared to MAML1.

As mentioned above MAML1 is involved in oncogenesis and in this respect we investigated to which extent MAML1 ubiquitination could play a role in tumor development. For this reason we screened the Catalogue of Somatic Mutations in Cancer (COSMIC) database in an attempt to detect mutations in MAML1 in various cancer cell lines (Forbes et al. 2014). Several mutations in MAML1 were found, most of them are in the C-terminal of the protein, but none of them were a lysine residue involved in ubiquitination. Even though we were not able to identify any mutation in a lysine residue linked to ubiquitination during our search, we cannot exclude the possibility that any of the detected mutations could affect ubiquitination.

4.2 MAML1 INTERACTION WITH TRANSCRIPTIONAL AND EPIGENETIC REGULATORS

In order to further understand how MAML1 protein is regulated in Notch signaling, we investigated the functional interactions among Notch and the Notch coactivators MAML1, p300 and CDK8 (**Paper I**). We performed both reporter gene assays in cells and experiments based on an artificial cell-free transcription system. The data showed that when p300, CDK8 and MAML1 were present altogether, they more potently enhanced Notch transcription compared to a system where one of these factors is either not overexpressed or missing. These observations suggested that there is a functional cooperativity among MAML1, p300 and CDK8 in order to stimulate Notch dependent transcriptional activation. Even though a previous study has shown that CDK8 inhibits Notch activation *in vivo* and thus it reduces the expression of Notch targeted genes, it also plays an important role in controlling the nuclear levels of various transcriptional activators (Fryer, White, and Jones

2004; Bancerek et al. 2013; Donner et al. 2010). Therefore, CDK8 may affect Notch signaling in different ways according to the cell's needs

As mentioned above MAML1 interacts with p300, that comprises histone acetylase activity, and it is likely that MAML1 also interacts with other proteins involved in epigenetic regulation. In **Paper III** we studied the interaction of MAML1 with the transcriptionally repressive complex DNMT1-PCNA-HDAC2, which exerts its repressive activity by DNMT1-mediated DNA methylation and HDAC2-mediated histone deacetylation (Rountree, Bachman, and Baylin 2000). *In silico analysis* using IST database and Array Express data showed that *MAML1* levels correlated with *HDAC2* and *DNMT1* on the transcription level. Moreover, immunoprecipitation and GST-pull down assay data revealed that MAML1 protein interacts with HDAC2 and with a lower affinity with PCNA. However, no interaction was observed between MAML1 and DNMT1. Supporting data came also from the use of fluorescence microscopy, which showed a co-localization of MAML1 with DNMT1, PCNA and HDAC2 in HEK293 cells. Considering all the above we suggested that MAML1 may interact with the DNMT1-PCNA-HDAC2 repressive complex, but further studies are needed in order to determine its exact role in epigenetically regulated transcriptional repression.

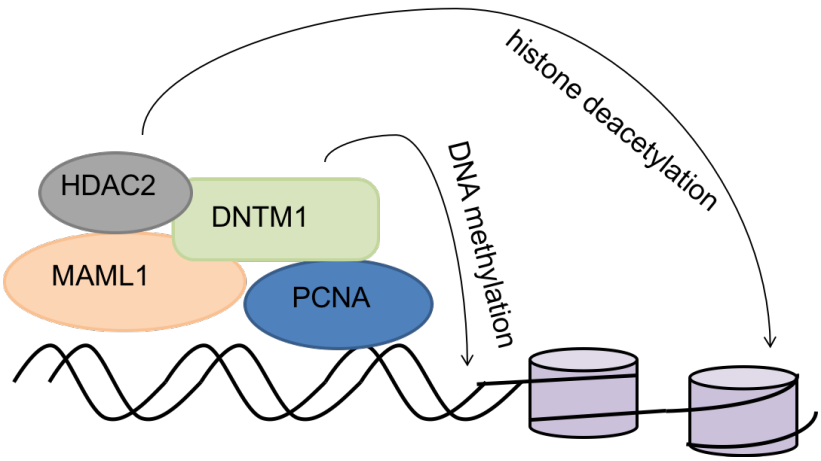

Figure 4.2 MAML1 possible interaction with the repressive complex DNMT1-PCNA-HDAC2

4.3 MAML1 INVOLVED IN CELL PROLIFERATION

Data on the involvement of MAML1 in tumor development, which is a disease linked to cell proliferation, are constantly accumulating (see **§1.7**) and considering the interaction of MAML1 with PCNA, a cell proliferation marker, we studied in **Paper II**, whether MAML1

is involved in cell proliferation (Kubben et al. 1994). *In silico* analysis based on normal human kidney samples revealed significant correlations between MAML1 and PCNA, CDK2 and XRCC5 (Ku80), the last two also being known proliferation markers (Li, Nelsen, and Hendrickson 2002; Spencer et al. 2013). Cell proliferation with HEK293 cells in which MAML1 had either been overexpressed or knocked-down showed that overexpression of MAML1 increased proliferation rate, whereas MAML1 knock-down resulted in a reduced cell proliferation. Therefore we suggested that MAML1 is involved in HEK293 cell proliferation.

4.4 ARSENIC REDUCES MAML1 PROTEIN LEVELS AND HEK293 CELL PROLIFERATION

Since MAML1 is involved in kidney cell proliferation and arsenic can accumulate in kidney cells (see **§1.3)**, we further studied whether arsenic affects MAML1 protein and subsequently cell proliferation in kidney cells (**Paper II**). For this purpose, we treated HEK293 cells with 1μM and 5μM of sodium meta-arsenite and quantified MAML1 mRNA and protein levels. We observed a significant reduction in the protein levels at 5μM of arsenic, whereas a reduction in mRNA levels could be detected already at 1μM of arsenic. Moreover, microscopic observations revealed a reduced number of cells at 5μM compared to control and 1μM. We performed flow cytometry in order to investigate whether this change in the cell number was due to apoptosis induced by arsenic treatment. We could not detect any increase in the apoptosis after 24 hours of arsenic treatment with either 1μM or 5μM of arsenic. In an additional step, we explored cell proliferation in arsenic treated HEK293 cells. Interestingly, we observed a reduction in cell proliferation after 2-3 days of 5μM arsenic exposure. In summary, we suggested that arsenic can suppress embryonic kidney cell proliferation through a decrease in MAML1 protein levels, but this does not include apoptotic or necrotic cell death. Instead, we speculate that arsenic induces autophagy in this cell line, which can lead to non-apoptotic cell death (Okada and Mak 2004). Arsenic has previously been shown to induce autophagy in a human leukemia cell line (HL60) and has at the same time been associated with a suppression of apoptosis (Y. Yang et al. 2008). However, further experiments are needed in order to investigate whether autophagy is the mechanism behind the decrease in cell proliferation in the arsenic treated population.

4.5 ARSENIC EFFECT ON THE DNMT1-PCNA-HDAC2 COMPLEX

Having already observed that arsenic affects MAML1 levels (**Paper II**) and considering that MAML1 interacts with the DNMT1-PCNA-HDAC2 complex, we further investigated the effect of arsenic on the proteins in this repressive complex by exposing HEK293 cells to 1μM and 5μM of arsenic (**Paper III**). We observed that arsenic increased PCNA and decreased DNMT1 protein levels, whereas the levels of HDAC2 remained unchanged. Based on these findings, we suggested that arsenic, apart from MAML1, affects also the levels of DNMT1 and PCNA. Thus, we hypothesized that arsenic may inhibit the formation or decrease the activity of this complex and thereby affect gene expression. However, further experiments are needed in order to confirm this hypothesis.

4.6 ARSENIC SPECIES DETECTED IN HEK293 CELLS

An important question when treating HEK293 cells with arsenic is whether the cells perform arsenic methylation, a detoxicfication process during which inorganic arsenic turns into the less reactive dimethylarsinic acid (DMA), through the intermediate state of methylarsonic acid (MMA), which is considered to be more reactive (Petrick et al. 2000). In **Paper II**, we used HPLC-HG ICP-MS analysis to measure the concentrations of different arsenic species in HEK293 cells exposed to various concentrations of sodium meta-arsenite. Indeed, we could detect the main arsenic metabolites; methylarsonic acid (MMA) and dimethylarsinic acid (DMA) in the cell lysate, suggesting that arsenite was methylated, presumably by arsenic methyltransferase (AS3MT) (Schläwicke Engström et al. 2007). Moreover, the main part of arsenic detected in the lysate was in the form of DMA, indicating that arsenic methylation did occur within the cells. We also observed that most of the arsenic added in the cell culture was found in the cell medium, with about 10 % detected in the cell lysate and only a small proportion detected in the cell debris. Interestingly, at 1μM of exposure 70 % of the arsenic detected in the cell lysate was identified as bound after lysate digestion. For cells exposed to 5μM of arsenic very small amounts of bound arsenic were detected. This last observation is most likely due to the decreased cell number in the culture and the degradation process that follows cell lysis. The aforementioned data suggest that HEK293 cells can effectively methylate arsenic even at 5μM of exposure and that a big part of arsenic at 1 μM of exposure is bound to cell components, presumably proteins with sulfhydryl (SH) groups (S. Shen et al. 2013).

4.7 ARSENIC-RELATED EFFECTS ON H3K9ME3 AND H3K9AC IN LYMPHOCYTES

Since there are accumulating data on the effect of arsenic on the epigenome (see **§1.9**) and since we have observed that arsenic can affect the epigenetic regulation complex DNMT1-PCNA-HDAC2 in kidney cells, we further studied whether arsenic could affect H3K9me3 and H3K9Ac histone modifications (**Paper III**). For this purpose, we collected blood lymphocytes from women living in San Antonio de los Cobres in Argentina, sorted them in CD4+ and CD8+ fractions and quantified global H3K9me3 and H3K9Ac levels. We observed that women with high urinary arsenic concentrations (>128 μg/L, median urinary concentration) had significantly lower levels of global H3K9me3 in the CD4+ cells, compared to those with lower urinary arsenic concentrations (≤128 μg/L p=0.019). No differences were reported in H3K9me3 in CD8+ cells. To our knowledge, this is the first human study reporting an effect of arsenic on H3K9me3. H3K9me3 is a marker of constitutive heterochromatin, where silenced early developmental genes are situated and several studies have reported that re-expression of these genes can lead to tumor development, suggesting that changes in this epigenetic marker may lead to adverse health effects such as cancer (C.-S. Hong et al. 2015; Litvinov et al. 2014).

We observed no association between the women's urinary arsenic concentrations and H3K9Ac levels in any of the cells types. This is in contrast to, a previous study reporting a decrease in H3K9Ac levels with increasing urinary arsenic concentrations (Chervona et al. 2012). However, that study was performed on individuals from Bangladesh, whose median urinary arsenic concentration was lower than in our study, and different cell types were studied. They detected changes in histone modifications in whole blood, whereas in our study we sorted the blood cells and performed the analysis in CD4+ and CD8+ cells separately. A whole blood sample includes many different types of cell types, which may mask the effects of arsenic on the different cell types.

In order to study the effect of arsenic on histone modifications further (**Paper III**), we exposed two human CD4+ cell lines (Jurkat and CCRF-CEM) to 0.1 μg/L, 1 μg/L and 100 μg/L of arsenic *in vitro*. Our data showed an increase in H3K9me3 levels in both cell lines, although not statistically significant. Even though it was not possible to detect a consistent pattern with a statistical significance when it comes to H3K9me3, we observed that this marker was affected by arsenic in all the experiments by either an increase or a decrease in its global levels. A previous *in vitro* study has also reported an effect of arsenic on H3K9me3, showing an increase in H3K9me3 levels near the transcription start site of the

myogenin gene at 20nM of arsenic exposure (G.-M. Hong and Bain 2012). On the other hand, the levels of H3K9Ac increased with increasing arsenic concentrations in both cell lines at 1 μg/L. This finding is in accordance with a previous study showing an increase in H3K9Ac levels in hepatocarcinoma cells in response to arsenic exposure (S. Rahman et al. 2015). Since we observed changes in H3K9Ac global levels, we investigated whether this could be due to changes in HDAC2 levels (**Paper III**). We could not detect any statistically significant changes in HDAC2 levels in relation to arsenic exposure. However, an interesting observation was that in Jurkat and CCRF-CEM cells the low levels in HDAC2 reported at 48 h of exposure correlated with an increase in H3K9Ac levels at 72 h of exposure at 0.1 μg/L and 1 μg/L of arsenic, respectively. We thus speculated that the arsenic-related decrease in HDAC2 levels may have led to the increase in H3K9Ac levels. Ramirez *et al.* reported a similar effect in HepG2 cells, where inhibition of HDAC activity by arsenic increased H3K9Ac global levels (Ramirez et al. 2008). Considering all the above we suggested that arsenic exposure *in vitro* affects the trimethylation and acetylation of H3K9 in CD4+ cells, potentially via HDAC2 activity.

4.8 METHODOLOGICAL CONSIDERATIONS - LIMITATIONS OF THE STUDY

In **Paper I** and **Paper II** we used artificial systems in order to study MAML1 protein. Since natural MAML1 levels in the cells are quite low and thus harder to detect, we overexpressed both MAML1 and the proteins N1ICD, p300 and CDK8 in immortalized cell lines. We also used an artificial system, an *in vitro* transcription assay, in order to study MAML1 direct interactions with p300 and CDK8. The use of such systems is a limitation of the study, since an artificial system does not mimic the systems in the cell and thus the data may not mirror the exact signaling processes that occur in the cell.

In **Paper II and III** we used an immortalized cell line (HEK293) in order to study the effect of arsenic on MAML1, DNMT1 and PCNA. HEK293 cells are of human embryonic kidney origin and they have been immortalized with the use of sheared adenovirus 5 DNA (Kavsan, Iershov, and Balynska 2011). Although HEK293 cells are easy to grow and transfect, they do not exactly mirror normal or cancerous human cells. Thus, the data produced from such a system can provide important information, but should be interpreted with caution regarding their resemblance to the natural processes in the cell.

In **Paper III** we used the immortalized cell lines Jurkat and CCRF-CEM. The CCRF-CEM cell line is derived from a patient with acute lymphoblastic leukemia (ALL), whereas the Jurkat cell line is derived from a patient with a subtype of ALL, acute T cell leukemia

(ATLL). In both cases the cells are of tumor origin and thus they host many different genetic and epigenetic alterations compared to normal blood cells. In this respect, it is important, as mentioned above, to be careful when interpreting data derived from these cell lines regarding their resemblance to natural processes.

Furthermore, in **Paper III** we isolated lymphocytes from arsenic exposed individuals in order to investigate histone modifications in relation to arsenic exposure. The number of individuals included in the study was 28, which is too small to draw any definite conclusions. Thus, the low number of samples could be the reason that we could not detect any statistically significant changes in H3K9Ac global levels or that we have overestimated the changes in H3K9me3 levels. In this respect, even though our data provide an important hint towards the effect of arsenic on histone modifications, further studies are needed in order to draw a more definite conclusion.

5 GENERAL DISCUSSION

The studies presented in this thesis explore the transcriptional regulation via MAML1 protein, a transcriptional co-activator, and the potential effect of arsenic on both MAML1 and the MAML1-interacting proteins DNMT1, HDAC2, PCNA, as well as on histone post-translational modifications. As suggested by the data, MAML1 is an important part of Notch signaling and its levels in the cell are regulated through ubiquitination. I addition, interactions of p300 and N1ICD with MAML1 appear to affect the degradation of MAML1 by stimulating or reducing its ubiquitination respectively. Moreover, MAML1 mutations were observed in many different human cancer cell lines, though we were not able to link them to MAML1 ubiquitination. We also report that at least in the human embryonic cell line (HEK293) MAML1 is involved in cell proliferation. We also report that arsenic downregulates MAML1 levels in HEK293 cells resulting in decreased cell proliferation. Arsenic, apart from MAML1, affects DNMT1 and PCNA levels in the cell, both of which are members of the DNMT1-PCNA-HDAC2, a transcriptional and epigenetic regulatory complex, which we report that interacts with MAML1. We also report that arsenic, apart from affecting the aforementioned epigenetic complex, further influences epigenetic regulation by decreasing H3K9me3 global levels in CD4+ cells isolated from arsenic exposed women in Argentina and increasing H3K9Ac global levels in T lymphocytes *in vitro*.

Arsenic is both a toxic metalloid and a carcinogen. Several studies have focused on its effects on the human body after chronic consumption through food or drinking water (M. M. Rahman, Ng, and Naidu 2009; Y. Chen and Karagas 2013; Wang et al. 2009; Ahmed et al. 2012). For example, Ahmend *et al*. reported in 2014 that arsenic appears to affect the cell-mediated immune response of children exposed to it pre- and postnatally (Ahmed et al. 2014). In **Paper III** we reported that arsenic can affect T lymphocytes' epigenome by decreasing H3K9me3 global levels. T lymphocytes are an important part of cell-mediated immunity and deregulation of their function may result in the development of various diseases, like autoimmunity and cancer (Aifantis, Raetz, and Buonamici 2008; Dornmair et al. 2003; Femke Broere , Sergei G. Apasov, Michail V. Sitkovsky 2011). However, we observed no changes in the levels of H3K9Ac in the Argentinian arsenic exposed women, which is in contrast with what was reported by Chervona *et al.* in Bangladeshi women (Chervona et al. 2012). As mentioned before the two studies differ not only to the human population studied, but also to the type of samples that were analyzed. A conclusion that can be drawn by this comparison is the importance of separating the cells from a blood sample in

order to investigate possible epigenetic changes in the human population. The reaction to environmental changes can be cell type specific and thus an analysis of a mixed population of cells may mask changes that take place in individual cell types (Simkó 2007).

Another important observation that was made in **Paper III** was the very low arsenic concentrations that could lead to changes in the global levels of histone modifications studied *in vitro*. Many studies have reported the adverse effects of arsenic exposure on the human body (Hughes et al. 2011; Argos et al. 2010). From acute toxicity to cancer development most of these studies report arsenic concentrations higher than 10 μg/L, which is the drinking water limit suggested by WHO ("Guidelines for Drinking-Water Quality. Third Edition. - WHO - OMS -" 2016). The arsenic concentrations used in the *in vitro* study are similar or below the levels reported in the blood of the non-exposed women (0.69-1.8 μg/L) that were used as a control in the population study, part of which was the study described in **Paper III** (Concha, Nermell, and Vahter 1998). Such low arsenic concentrations could be a result of arsenic consumption through food, e.g. rice, cereals, which is common in many parts of the world (Sand et al. 2015; Meharg et al. 2009). Although it is not possible to draw conclusions for the human organism from an *in vitro* study, as an organism is more complicated and is composed of many different cell types compared to a monoculture in the lab, it is still important to further study the effect of such low arsenic concentrations on the population in order to protect human health.

Furthermore, in **Paper III** we report changes in DNMT1 and PCNA protein levels, both parts of the DNMT1-PCNA-HDAC2 complex and thus the epigenetic machinery, in kidney cells in relation to arsenic exposure (Rountree, Bachman, and Baylin 2000). Changes in the levels of these two proteins could possibly affect the formation or the activity of the repressive complex and thereby disturb the epigenetic profile of the cell. Many studies have reported changes in the DNA methylation levels or PTHMs levels in populations exposed to arsenic (J. R. Pilsner et al. 2007; Chervona et al. 2012). Even though further studies need to be performed in order to investigate in more detail the DNMT1 and PCNA protein levels deregulation in relation to the epigenome, the present studies provide an indication that changes in the epigenome may be a result of changes in complexes responsible for the establishment of epigenetic marks.

Research on arsenic so far has led to many contradictory data, especially when studying the epigenome. Pilsner *et al.* reported an increase in DNA global methylation in relation to arsenic exposure in human leukocytes, whereas Chanda *et al.* observed both hypermethylation and hypomethylation in specific gene promoters in blood cells in relation to

arsenic exposure (J. Pilsner et al. 2011; Chanda et al. 2013). Similar examples can be found from studies focused on individual protein levels or transcription (G.-M. Hong and Bain 2012; S. Shen et al. 2013). Even though one could argue that these differences could be attributed to the different experimental setups, which could absolutely be the case, arsenic has been shown to increase DNA double-strand breaks and as we mentioned above affect proteins involved in epigenetic regulation (Bustaffa et al. 2014). In this respect arsenic may cause epigenetic instability in the cells and thereby affect the various proteins in a stochastic way, which has previously been shown for cadmium (Vilahur, Vahter, and Broberg 2015). Epigenetic instability can change chromatin's structure and localization in the nucleus and lead to expression of silenced genes or repression of active ones (Putiri and Robertson 2011).

To this point the main focus was on the negative impact of arsenic on the human health, but arsenic has been known since ancient times also for its curative effects (Waxman 2001). In 1996 Chen *et al.* suggested that arsenic could be effective against APL cells and a clinical trial performed in 1997 validated the curative effect of arsenic on APL (G. Q. Chen et al. 1997; G. Q. Chen et al. 1996). In **Paper II** we suggested a possibility of arsenic been effective against renal cancers overexpressing MAML1 protein. An *in silico* study has showed that MAML1 expression was increased in renal cancer cell carcinoma and that it correlated with decreased patients' survival (Hansson et al. 2012). In this respect, treatment of renal tumor cells overexpressing MAML1 with arsenic could possibly downregulate the levels of MAML1 and decrease cell proliferation, as was reported for HEK293 cells in **Paper II**.

6 CONCLUSIONS

The studies included in this thesis provide an insight on the effect of arsenic on transcriptional and epigenetic processes. We suggest that MAML1 protein, a transcriptional co-activator, is involved in cell proliferation and epigenetic regulation in HEK293 cells, through its interaction with DNMT1-PCNA-HDAC2 complex, which has the ability to repress transcriptional activation by establishing epigenetic marks on chromatin. We also report that MAML1 protein levels are regulated by ubiquitination, a process that as we show is enhanced by the presence of p300 and decreased by Notch1 ICD. We suggest that MAML1 functionally interacts with p300 and CDK8 in order to enhance Notch-mediated transcriptional activation. Once we identified MAML1 protein levels' regulation via ubiquitination and the possible involvement of MAML1 in epigenetic regulation, we further studied the effect of arsenic on this transcriptional co-activator and the MAML1-interacting proteins DNMT1, PCNA and HDAC2. We suggest that arsenic exposure affects the levels of MAML1, DNMT1 and PCNA in HEK293 cells and reduces cell proliferation probably via MAML1 downregulation. We additionally studied the epigenetic effects of arsenic on T lymphocytes. We show that arsenic decreases both H3K9me3 levels in CD4+ cells in the human population and H3K9Ac in T lymphocytes *in vitro*. We also propose an interplay between global levels of HDAC2 and H3K9Ac global levels in arsenic exposed T lymphocytes *in vitro*. All these findings suggest that arsenic affects both transcription and epigenetic regulation in cells, which could lead to long-term implications, like decreased immunity and cancer, on human health.

7 MAIN FINDINGS

- MAML1 protein levels are regulated via ubiquitination, which is stimulated by p300 and repressed by N1ICD.
- MAML1 interacts with p300 and CDK8 in order to stimulate Notch-mediated transcriptional activation and with DNMT1-PCNA-HDAC2 complex as part of its possible involvement in epigentic regulation.
- MAML1 is involved in cell proliferation in kidney cells.
- Arsenic exposure reduces kidney cell proliferation, possibly via MAML1 downregulation. Arsenic affects MAML1-interacting proteins DNMT1 and PCNA, which are involved in epigenetic regulation, by decreasing and increasing their protein levels respectively.
- Chronic arsenic exposure appears to decrease the global levels of the epigenetic mark H3K9me3 in CD4+ in an Andean human population.
- Arsenic decreases the global levels of the epigenetic mark H3K9Ac in T lymphocytes *in vitro*.

8 FUTURE RESEARCH

- Investigate whether, apart from MAML1 gene expression, MAML1 ubiquitination is also affected by arsenic exposure, since we have already shown that MAML1 protein levels are regulated by ubiquitination and arsenic downreguates MAML1.
- Investigate whether the MAML1 mutations identified in the various cancer cell lines affect MAML1 ubiquitination. We reported in this thesis that we could not detect any mutations in the lysine residues involved in MAML1 ubiquitination in the tested cancer cell lines, but we detected mutations in many other residues, e.g. proline. Thus, further studies are needed in order to elucidate whether the mutations we detected may have a potential role in MAML1 ubiquitination.
- Elucidate further the effects of arsenic exposure on Notch signaling. Since arsenic exposure downregulates MAML1, which is an important co-activator in Notch signaling, further studies could be performed in order to elucidate the effect of arsenic in Notch signaling transcriptional activation and on MAML1-interacting proteins p300 and CDK8.
- Investigate whether MAML1 is involved in cell proliferation in other cell types apart from HEK293 cells, in order to gain a deeper understanding of the mechanisms driving cell proliferation.
- Investgate whether there are MAML1-independent ways via which arsenic can decrease cell proliferation. Even though we have suggested that downregulation of MAML1 decreases cell proliferation of HEK293 cells in the context of arsenic exposure, further studies are needed in order to investigate other pathways through which arsenic may affect cell proliferation.
- Investigate whether HEK293 cells exposed to arsenic undergo autophagy, since, as repoted in the present thesis, we observed reduced cell proliferation in the arsenic exposed cells, but could not detect apoptosis or necrosis.
- Investigate the possible role of MAML1 in epigenetic regulation through its interaction with DNMT1-PCNA-HDAC2 complex and whether arsenic exposure influences the activity of the DNMT1-PCNA-HDAC2 complex. We have shown that MAML1 interacts with DNMT1 and PCNA, but what is the effect of this interaction to epigenetic regulation needs to be further researched. Also, since arsenic exposure affects the epigenome in various ways and affects also MAML1, DNMT1 and PCNA

levels in kidney cells, future studies could investigate the effect of arsenic on the aforementioned epigenetic complex activity.

- Investigate further and in a larger population sample the effects of arsenic exposure on PTHMs in order to further elucidate the effects of arsenic on the epigenome.
- Investigate further whether the arsenic-related decrease in HDAC2 levels correlates with the increase in H3K9Ac levels, since we have observed a possible correlation between the two.
- Investigate further the effect of low arsenic concentrations on the PTHMs in human cells both *in vitro* and *in vivo*, as we reported in this thesis that even low arsenic concentrations may influence PTHMs and there is accumulating evidence that humans are exposed to low arsenic concentrations through food, in particular rice.
- Investigate whether arsenic exposure leads to epigenetic instability in the cells, since this could give us a deeper understanding on arsenic's mode of action once entering the cell.

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